

New SARS-CoV-2 infection in a Pet Cat with severe lung disease in Italy

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ABSTRACT

The pandemic respiratory disease COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan in December 2019 and then spread throughout the world; Italy was the most affected European country. Despite the close pet-human contact, little is known about the predisposition of pets to SARS-CoV-2. Among these, felines are the most susceptible. In this study, a domestic cat with clear symptoms of pneumonia, confirmed by Rx imaging, was found to be infected by SARS-CoV-2 using quantitative RT-qPCR from a nasal swab. This is the first Italian study reporting on the request of the scientific community to focus attention on the possible role of pets as a SARS-CoV-2 reservoir. An important question remains unanswered: did the cat die from SARS-CoV-2 infection?

Keywords: SARS-CoV-2, cat, RNA-extraction, pneumonia

INTRODUCTION

The World Health Organization (WHO) declared the COVID-19 disease, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a worldwide pandemic [1]. The first epidemic cluster exploded in China, specifically in Wuhan city [2-3], as early as 1 December 2019. As of mid-July 2020, there have been over 5.525.245 confirmed COVID-19 cases worldwide, of which more than 30% cases in the EU and UK, with more than 347.108 global deaths [2] (<https://www.worldometers.info/coronavirus>. Accessed 10 May 2020). Specifically, Italy has been severely affected [4] and it was one of the first and hardest hit countries in Europe, with over 219.000 cases and 30.500 deaths reported [2], thus on 9 March 2020, a lockdown was declared for the entire country and progressively stricter restrictions were adopted [4,5].

The structure and the function of the SARS-CoV-2 are closely related to the already known coronaviruses responsible for the Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV). In detail, the mechanism of infection of the host cells is triggered by the external protein of the virus, the glycoprotein (S) peak, which is able to bind and recognize the angiotensin converting enzyme 2 (ACE2) of the human receptor, triggered by the serine of transmembrane protease type 2 (TMPRSS2), and/or extracellular matrix metalloproteinase inducer CD147 [2,6-8].

Because of the close contact between people and pets, such as dogs and cats, the scientific community started investigating the possibility of animal-human virus transmission [9]. Furthermore, animal ACE2 receptor has high human ACE2 aminoacidic sequence identity [10-12]. It has been shown that some animal species, particularly felines, can occasionally

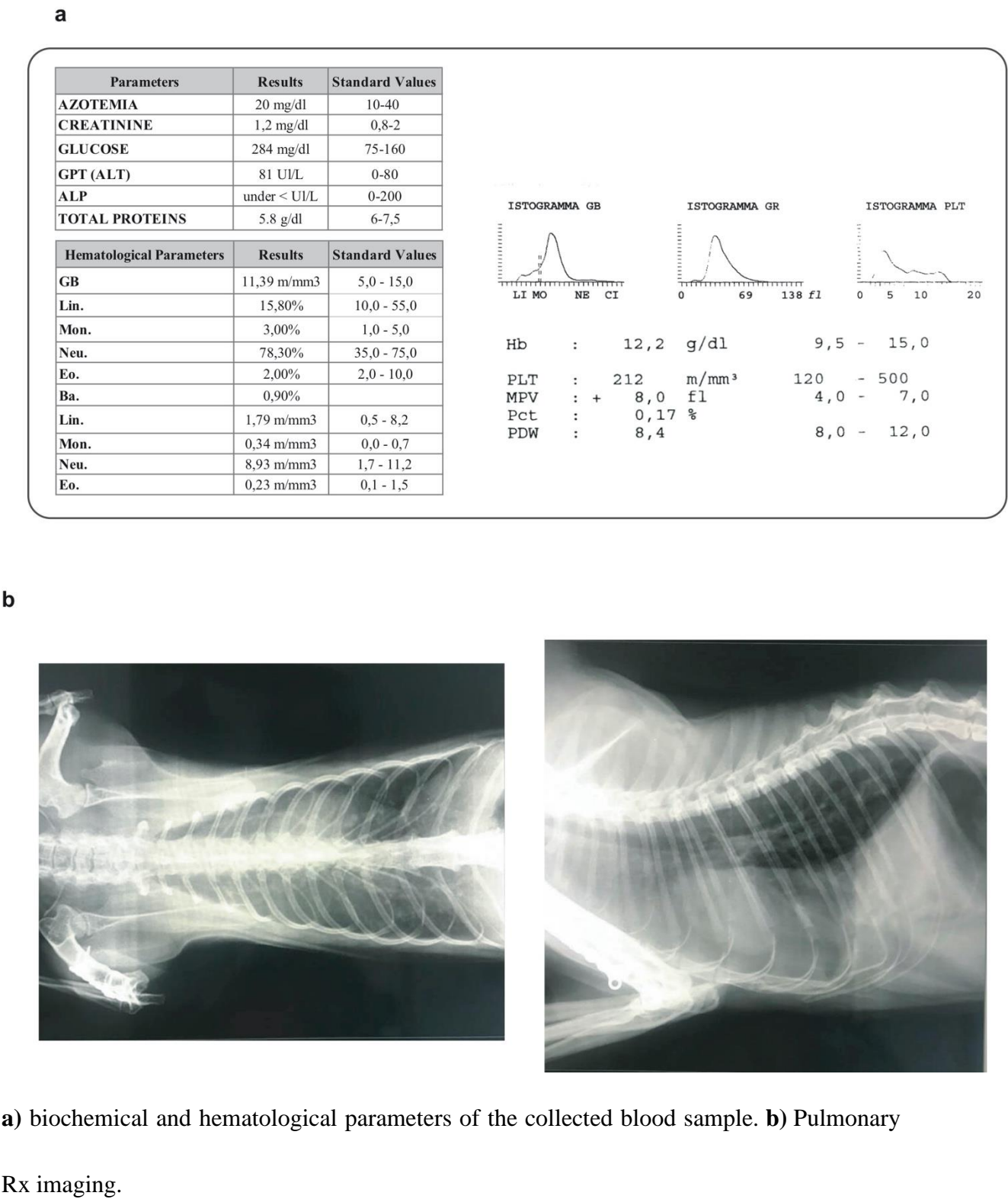
become infected with SARS-CoV-2 [10,13-15]. So far, some cases of domestic animal infection have been reported in Belgium [16], Hong Kong [17] and France [18], but no cases have yet been investigated in Italy. In particular, by introducing SARS-CoV-2 virus samples through the nasal cavities of domestic cats it was possible to find viral RNA and infectious viral particles in their upper respiratory tract, but none of the infected cats showed symptoms of the disease. In addition, viral RNA was detected in 1:3 healthy cats exposed to infected felines, suggesting that they contracted the virus from the droplets exhaled by infected cats [13,19].

In this study we identified, for the first time, the natural infection of a cat by SARS-CoV-2 in Italy. The RNA extracted from the feline nasal swab was processed by RT-qPCR revealing the presence of two SARS-CoV-2 genes; moreover, a part of the gene was further sequenced to evaluate its nature. In addition, we excluded the presence of feline infectious peritonitis (FIP), a lethal systemic disease often associated with feline coronavirus (FCoV).

VETERINARY CLINICAL CASE

A sterilized male European shorthair cat was presented to a veterinary clinic by the owner reporting serious respiratory distress of about three days. On physical examination, the cat had severe dyspnea and sialorrhea, Kussmaul breathing, asynchronous chest and abdomen. To confirm the suspicion of pneumoniae, a blood sample was collected from the jugular vein for hematological and biochemical analysis. Furthermore, given the similar symptomatology and the current situation caused by SARS-CoV-2, a nasal swab for SARS-CoV-2 was taken. The main biochemical and hematological parameters revealed lower alkaline phosphatase and higher glycemia values than normal, while the blood count test revealed a relative and absolute neutrophilia (Figure 1a). Rx imaging revealed interstitial pneumonia with an area of pulmonary opacity (Figure 1b) leading to the suspicion of spillage, excluded from the ECO-ultrasound examination (data not shown). Despite antibiotic therapy in combination with aerosol the cat died during the night.

Figure 1. Feline clinical parameters and Rx.



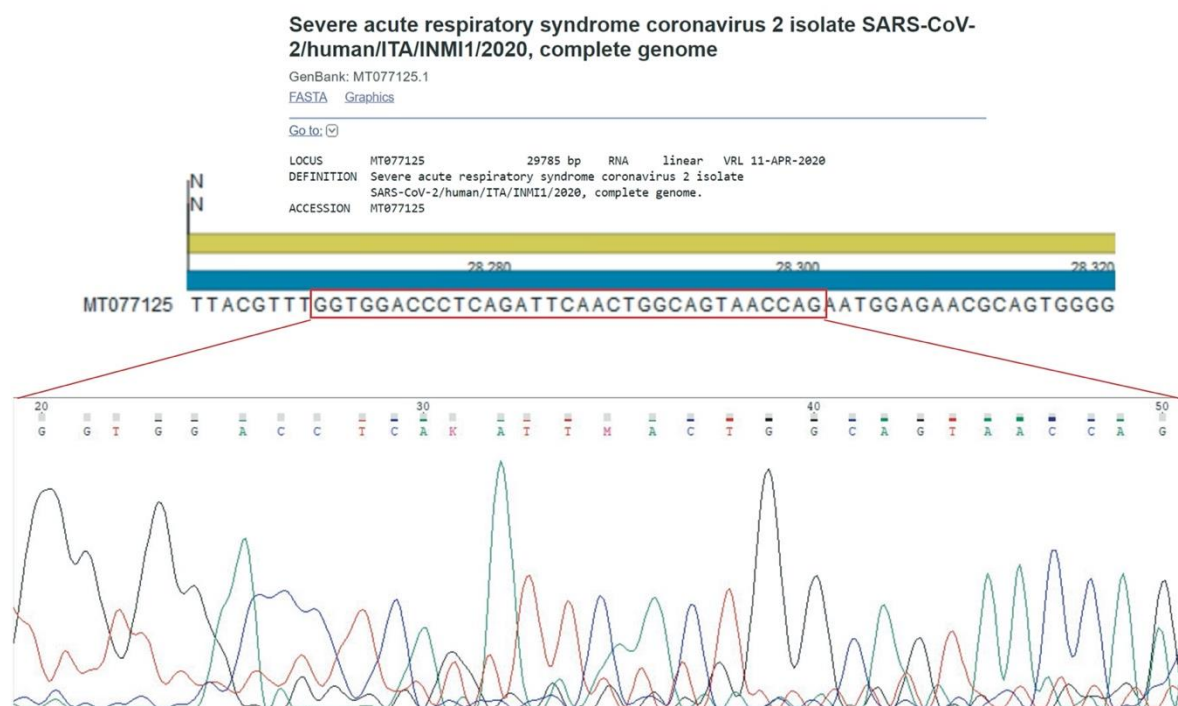
RESULTS

RNA extracted from the feline swab was quantified as 6,56 ng/μl and was tested in duplicate by TaqMan Probes for the detection of SARS-CoV-2 N1 Portion Gene. SARS-CoV-2 synthetic RNA and feline swab RNA amplified within the 28th and 34th cycle threshold (CT), respectively, while no amplification for the No Template Control (NTC) was detected. The amplification curves and CT, average and SD data are reported in Figure S1a (Supplementary materials). Furthermore, the real-time qPCR products ran on a 1,8% agarose gel to confirm the correct size of the amplicon (Figure S1a, right).

Another piece of evidence of the SARS-CoV-2 presence was given by the detection of the spike gene within the 40th CT during the real-time qPCR assay, while no amplification for SARS-CoV-2 synthetic RNA and NTC was detected (Figure S1b). Real-time qPCR using TaqMan Probes was performed to exclude the presence of Feline Infectious Peritonitis (FIP), a lethal systemic disease often associated with feline coronavirus (FCoV), comparing our feline sample to another feline positive for FIP. The absence of amplification confirmed the negativity of our sample for FIP; on the other hand, the FIP Control Kit and feline FIP Positive Sample amplified within the 20th and 38th CT, respectively (Figure S1c). To ensure that two different real-time activities were conducted on the same RNA during the sample collection and processing phases, so as to exclude the presence of any cross-contamination, the feline housekeeping gene (Techne-FCoV Kit) was chosen to verify the presence of correct feline RNA. The feline swab sample amplified within the 39th CT, while there was no amplification for the NTC (Figure S1d). At the same time, the feline sample was negative for human beta-actin that was used as control.

Finally, the amplified fragment obtained by an end-point PCR using the same RT-qPCR primers (Figure S1a, right) was sequenced using the Sanger method to verify the accuracy of the amplification, resulting in a perfect match with the SARS-CoV-2 N1 gene according to the BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Figure 2).

Figure 2. Sequence of amplified end-point PCR derived fragment compared with SARS-CoV-2 genome. The amplified fragment was obtained by an end-point PCR using the same RT-qPCR primers and had the same sequence as the SARS-CoV-2 N1 gene.



DISCUSSION

In late-December 2019, severe acute respiratory syndrome–coronavirus 2 (SARS-CoV-2), identified as a novel coronavirus [20-31], caused an uncommon pneumonia in humans in Wuhan (China), first, and then rapidly extent internationally. Thus, the World Health Organization (WHO) designated the disease caused by this virus as coronavirus disease 2019 (COVID-19) and officially declared COVID-19 a pandemic [32]. There is evidence that SARS-CoV-2 shares 96,2% of its nucleotide identity with the coronavirus RaTG13, detected in horseshow bats in China [33]. Indeed, first indications revealed that the infection occurred through the consumption of meat derived from infected bats, of which the Chinese are consumers. Despite this, the question if SARS-CoV-2 could be transmitted to other animal species that could then become a reservoir of infection was not immediately a priority but became relevant later as pets - such as cats, dogs and ferrets - are in intimate contact with humans. Thus, the knowledge of their susceptibility to SARS-CoV-2 is very important and several cases of infected pets have been reported. Unlike several studies carried out on dogs [13], which have shown a low, if not absent, susceptibility to the virus, felines - especially cats - seem to have a greater predisposition to acquire the infection [13].

In the same way as other coronaviruses, such as SARS-CoV, the feline coronaviruses described until now seem to be able to switch their tropism, developing several specific virulent pathotypes that could even cause cross-species transmission [34]. Since the beginning of the pandemic, several cases of infected cats have been reported, although it is still not clear how the infection occurred and what type of symptoms is present [18].

In this study, we report - for the first time in Italy - the case of a male European cat with clear symptoms of respiratory distress probably correlated to SARS-CoV-2. The analysis of a blood sample from the cat and the subsequent X-Ray and Ultrasound investigation confirmed the diagnosis of severe pneumonia. As cat's pathology evolved rapidly and unusually harmful (the animal died in only 3 days), with symptoms and rate of disease progression similar to patients with COVID-19, it was decided to perform a nasal swab in order to verify a possible infection from SARS-CoV-2. The analysis of RNA extracted from the feline swab through five different real-time tests allowed us to exclude the presence of related pathologies - such as the classic FCoV - or contamination from other species (i.e. humans). Interestingly, the identification of the SARS-CoV-2 virus was supported not only by the amplification of the commonly used N1 gene within the 34th CT, by means of the TaqMan probe, but also by the amplification, using a more economic SYBR green chemistry, of another portion of the SARS-CoV-2 virus spike gene – missing in the synthetic RNA commonly used as positive control - that can represent a new molecular identification target. The negative result of real-time amplification of the feline sample with the Taqman probe for FCoV, excluded the possible positivity to a classic feline Coronavirus, responsible for FIP. At the same time, the feline sample was cross-contamination free, confirmed by a positive amplification for a feline housekeeping gene and by the absence of amplification of the human housekeeping gene beta-actin. Finally, to verify the presence of SARS-CoV-2, an end-point PCR was performed with CDC primers targeting N1 and the products were subsequently sequenced using the Sanger method.

Although the clinical history and the experimental path give a clear result on a positivity with lung symptoms for SARS-CoV-2, the infection source could not be clarified. Probably, the contamination occurred through a third way determined by the feline habit of licking surfaces, hypothetically contaminated, or by contact, not detected by the owners, with other not identified positive cats. No studies have yet been carried out that demonstrate a possible human-animal transmission. At the same time, the presence of the SARS-CoV-2 in the feline sample alone is not enough to assert that it was the only cause of the pathological event [35]. However, it was likely one of the possible components responsible for progressive aggravation of the cat's health and its consequent death. To date, the crisis phase in some parts of the world seems to be over, but the natural reservoirs of the virus and its high contagion rate, as well as environmental and social conditions, could allow it to come back as a second wave. Particularly, as cats – but also pets and farm animals - are more common and in closer contact with humans than bats and seem to be a natural reservoir for the virus, they should be checked for SARS-CoV-2 when affected by severe pneumoniae. However, the question about the linkage between the SARS-CoV-2 infection and the cat death remain unsolved.

MATERIALS & METHODS

Specimen collection

A feline nasal swab was collected with the Virus Test Kit Diagnostics Sterile Pack Swabs Universal Viral Transport System (COD. RYCO-VART10B03, Jiangsu Rongye Technology Co., Ltd, Touqiao Town, Yangzhou City, China).

RNA Extraction

To increase the RNA uptake, a variation to the standard protocol was performed: 700 µl of swab buffer was processed in the same column, instead of the original 140 µl. AVE Buffer and ethanol were added at the same proportions in order to reach a final extraction volume of 6,3 ml. Then, the total volume was eluted in the same column 10 times. The rest of the extraction process was performed according to the protocol provided by the manufacturers. Purification of viral RNA from the original swab was performed using QIAamp® Viral RNA minikit (COD. 52904 QIAGEN, Hilden, Germany).

RNA Quantification

Extracted RNA was quantified by the fluorimetric technique using the Qubit™ RNA HS Assay Kit (Thermo Fisher, Catalog number: Q32852) according to the standard procedure.

RNA reverse transcription

Feline nasal swab RNA reverse transcription was carried out by using the QuantiTect® Reverse Transcription kit (QIAGEN, Hilden, Germany).

Real-time RT-qPCR

RT-qPCR targeting the SARS-CoV-2 was performed with TaqMan chemistry. The 2019-nCoV_N1 forward primer (5'-GAC CCC AAA ATC AGC GAA AT-3'), the 2019-nCoV_N1

reverse primer (5'-TCT GGT TAC TGC CAG TTG AAT CTG-3') and the 2019-nCoV_N1 probe (5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3') were used for the N gene assay [36,37].

The TaqMan® probe was labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3'-end with the Black Hole Quencher 1 (BHQ-1) (Eurofins Genomics); the reaction was performed with QuantiNova Probe PCR (Qiagen 208252) according to the manufacture's recommendations. To exclude the presence of FIP, we used the Techne® qPCR Kit for feline Coronavirus (FCoV) genomes (Techne®, USA) based on the TaqMan® principle. The reaction mixture, 15 µl, was made up of OneStep 2x qRT-PCR MasterMix (10 µl), FCoV primer/probe mix (BROWN) (1 µl), internal extraction control primer/probe mix (BROWN) (1 µl) and RNase/DNase free water (WHITE) (3 µl), and was used according to the handbooks provided by the manufactures. The thermal cycle was performed at 55 °C for 10 min for reverse transcription, followed by 95 °C for 3 min and then 45 cycles of 95 °C for 15 s and 58 °C for 30 s. To amplify another portion of SARS-COV-2, the 2019-nCoV spike forward primer (5'-CGGCCTTACTGTTTTGCCAC-3') and 2019-nCoV spike reverse primer (5'-TGTACCCGCTAACAGTGCAG-3')

were designed on the MT192773.1 sequence by Primer3 online software and used for the spike gene assay. Finally, the RT fragments were verified by running the RT products on 1,8% Agarose gel stained with (CANVAX) Greensafe DNA gel Stain (Cod E0206) and FastRuler Ladder (Cod SM1103). To exclude Human mRNA cross-contamination, Human Beta-Actin Primers were used as a control (Quantitech Primers, QIAGEN Cat No: QT00016786).

PCR amplification and sequence analysis

Amplicons obtained with PCR, from the amplification with the N1 portion gene primers (without probe), were purified using the QIAquick PCR Purification Kit (Cat. No.28106, Qiagen) and quantified by the fluorimeter Qubit dsDNA BR Assay Kit (Cat. No.32850, Invitrogen), then 5 ng of the product was sequenced on a SeqStudio Genetic Analyzer (Thermo Fisher Scientific) using the Applied Biosystems BigDye terminator cycle sequencing 3.1v (Cat. No. 4337455, Thermo Fisher Scientific) as previously described [38]. The Qubit dsDNA BR Assay Kit was used to quantify the product. It was then sequenced on a SeqStudio Genetic Analyzer (Thermo Fisher Scientific) using the Applied Biosystems BigDye terminator cycle sequencing 3.1v (Cat. No. 4337455, Thermo Fisher Scientific) as previously described [32] and compared with the reference sequence “MT077125 Severe acute respiratory syndrome coronavirus 2 isolated SARS-CoV-2/human/ITA/INMII/2020 (complete genome sequence release date: 11-APR-2020)” using the BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Ethical disclosure: ethical approval was not necessary as per institutional and national guidelines and regulations.

Author Contribution Statement: NM, AC and GE conceived and designed the study.

NM, AC and FA performed the experiments. SS, RV, SLS and AF contributed to the data analysis. NM and AC wrote the draft manuscript, SS reviewed and edited the manuscript.

All authors contributed substantially to the work, read and accepted the final version for the submission.

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Conflict of interest: None declared.

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